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THERMAL STABILITY OF IMMOBILIZED ENZYMES, AND SOME PRACTICAL APPLICATIONS

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Introduction

As a result of the development and application of upto-date biotechnological methods during the past twenty years, together with utilization of the possibilities available in the fermentation industry, new prospects have emerged for the fermentation industry, new prospects have emerged for the preparation of numerous organic compounds. This has led to a "biological explosion"; it can be ascribed to the crisis situations facing mankind as a consequence of the rapid growth in the world's population and of the industrial revolution: food and energy crises, environmental catastrophes and to a certain extent the shortage of raw materials. Biotechnology is currently seeking a solution to many of these problems, which are at present impeding scientific and technical development.

Enzyme technology is one of the most intensively developing branches of biotechnology; it is closely connected with bioreactor planning and gene manipulation methods. Its aim is enzymatic biotransformation, involving the preparation of organic compounds, in one at most only a few steps. The main advantages of enzymatic reactions is their high degree of specificity and the mild reaction conditions. However, there are obstacles to the practical application of enzymes in dissolved form: in many cases their stability is not satisfactory, and additionally they themselves remain as contamination in the reaction mixture at the end of the reaction they catalyse. These problems may be eliminated by the use of immobilized enzymes: following separation of the products, such enzymes may be used again and the transformation may be made continuous, which decreases the costs of enzyme utilization.

The possibility of the continuous use of immobilized enzymes is primarily governed by two conditions: the planning of a suitable bioreactor and the stability of the immobilized enzyme. The steric structure of enzymes is basically stabilized by the the intramolecular interactions determined by the primary structure (MOZHAEV and MARTINEK, 1984), further contributions being made in vivo by interactions with the cellular protein and nonprotein components. Numerous enzymes are rather unstable in isolated form, which limits their practical application. The stability may be increased by making the globular structure more rigid through covalent modification (TORCHILIN et al., 1978; GERMAIN et al., 1989) and through immobilization of enzyme isolated from a source with favourable thermal stability.

Enzyme molecules can not be regarded as a homogeneous population with completely uniform structure: the steric structures and amino acid compositions of the individual molecules may differ slightly (COLVIN, 1954). Consequently, the individual proteins in the heterogeneous molecules are inactivated at different rates in response to heat treatment, i. e. the kinetics of thermal inactivation is not of first order. KAWAMURA et al. (1981) developed a model to characterize the thermal denaturing of immobilized α-chymotrypsin, which was described as a large number of parallel, independent, first-order reactions. On this basis, MALHOTRA and SADANA (1987) and HENLEY and SADANA (1989) considered that the molecular and stability properties of the microheterogeneous enzyme population exhibit a continuous distribution, and they devised a graphical thermal inactivation model which can be fitted well to the experimentally determined thermal inactivation time curves. By this means it is possible to calculate the average value of the activation energy, and also the standard deviation in the activation energy, which characterizes the degree of microheterogeneity, i. e. the thermal stability of the enzyme.

The stability of immobilized enzymes in generally described on an empirical basis: too few data are available as concerns the theoretical basis for it to be stated which factors determine the stability. Our research group has prepared a number of immobilized enzymes for practical purposes, the stability playing the determining role. With this research background, therefore, I began a study of the factors influencing the thermal stability of enzymes. The aims related to the following four areas:

1. A comparative investigation of the stability features of mammalian skeletal muscle aldolases, and selection of the enzyme with the highest thermal stability, which is most suitable for immobilization.
2. On the immobilization of enzymes with different molecular properties on supports with different physical and chemical properties, establishment of general regularities as concerns the catalytic and stability properties of the immobilized enzymes.
3. Numeral analysis of the complex thermal inactivation time curves, and hence study of the intra- and intermolecular changes influencing the conformational state of the immobilized enzymes.
4. Utilization of the immobilized enzyme reactors for analytical and preparative purposes.

Methods

Aldolase was isolated from pig and rabbit skeletal muscle, and triosephosphate isomerase from pig skeletal muscle, by literature methods.

The enzymes were immobilized via covalent bonding on Akrilex C—100 (polyacrylamide-based, with carboxylic functional groups), Akrilex AH—100 (with acid hydrazide functional groups), Akrilex P—100 (activated with p-benzoquinone) and Sepharose 4B (activated with cyanogen

bromide) supports, and also on inorganic, silica-based supports activated with glutaraldehyde or p-benzoquinone (Silochromes).

A study was made of the thermal stability of the enzymes immobilized on the supports with different physical and chemical properties. The complex thermal inactivation time curves, consisting of activation and inactivation stages, were analysed by two methods. The changes in K_m and V_{max} were measured in the activation stage of heat treatment. In the inactivation stage, a numerical method was used to determine the activation energy of inactivation, its rate constant, and the standard deviation in the activation energy, indicative of the microheterogeneity of the immobilized enzyme, based on the graphical model of HENLEY and SADANA (1989). The role played in the thermal stability by the secondary interactions between the enzyme and the support was investigated on the basis of the effects exerted on the thermal stability by the pH, ion and protein concentrations, and the substances binding specifically or non-specifically to the enzyme molecule.

On an Akrilex C—100 support activated with disubstitued carbodiimides with various structures and molecular dimensions, a study was made of how the presence of the activating agent on the surface of the support influences the coupling and orientation of the enzyme molecules.

The immobilized enzymes were used to solve various analytical and preparative tasks in a continuous, column or batch reactor. The enzymes immobilized on the inorganic, Silochrome supports were employed as bed. Fructose-1,6-diphosphate (FDP) was determined in the immobilized aldolase-triosephosphate (TPI)-glycerophosphate dehydrogenase (GDH) enzyme reactor, and glucose in the immobilized glucose oxidase (GOD)-peroxidase (POD) reactor, in a flow injection system. In the aldolase-triosephosphte isomerase reactor, dihydroxyacetone phosphate was prepared; glyceraldehyde-3-phophate was separated from the main product on Dowex 1×2 anion exchanger. In the glucose oxidase-catalase batch reactor, glucose was oxidized to gluconic acid.

Results

1. Pig and rabbit skeletal muscle aldolases differ substantially in their stability to heat and denaturing agents. The conformational stability of pig aldolase is higher than that of the rabbit enzyme. The two enzymes differ as concerns the pH optimum of the thermal stability and the thermal inactivation time curve. The difference in stability is justified by the difference in stability is justified by the difference in amino acid sequence, which exists despite the great structural homology of the enzymes of these two phylogenetically close species. On an industrial scale, aldolase is cheaper to isolate from pig skeletal muscle.

2. Surprisingly, when pig skeletal muscle aldolase undergoes covalent immobilization on the polyacrylamide-based support bearing carboxyl functional groups, the active lysyl side chain is not modified, and the immobilized enzyme has a high specific activity decrease. This is due to two reasons. Not only the side-chains containing the amino groups, but also side-chains of other amino acids participate in the covalent binding. Secondly, there are steric effects as a consequence of the different structures of the supports. These may lead to considerable differences in the steric structure of the molecule. Similarly to aldolase, TPI can be immobilized well on the extremely hydrophilic Acrilex.

3. From the aspect of immobilization of GOD, with the prosthetic group, the less hydrophilic environment, i. e. the inorganic support, is the more favourable. On coupling, the orientation of the enzyme is influenced fundamentally by the chemical properties of the functional groups of the matrix.

4. In response to covalent immobilization, the thermal stability of the enzymes increases, and the rate of thermal inactivation is lower than that of the dissolved enzyme. Accordingly, they are suitable for the study of changes in conformation of molecules. The thermal inactivation time curves are complex; in many cases they consist of an activation stage, followed by an inactivation stage. The changes in V_{max} and $K_{m\ app}$ in the activation stage point to the changes in the steric structure of the enzyme molecule. The immobilized enzyme molecule is in a metastable state, and its conformation is influenced by secondary non-covalent interactions. The secondary interactions may develop between enzyme and support, and between enzyme and enzyme, and they may be affected by variations in the immobilized protein concentration, the ion concentration and the pH.

5. For most of the immobilized enzymes we investigated, the inactivation stage can not be discribed by first-order kinetics. This indicates that the covalently bound molecules comprise a large number of populations with different thermal stability. For numeral characterization of the thermal staility of these microheterogeneous system, we took the model of HENLEY and SADANA (1989) as basis and devised a method for calculation of the activation energy. The activation energy values calculated for the inactivation stage provide a good characterization of the enzyme thermal stability, while the standard deviations of the activation energy indicate that the immobilized enzymes have a higher degree of heterogeneity than that of the dissolved enzyme. The greater the microheterogeneity of the immobilized enzyme molecules, the greater the thermal stability.

6. The effects of factors influencing the thermal stability of the immobilized enzymes, and the extents of these effects, can be studied on the basis of the $K_{m\ app}$ and V_{max} values measured in the activation stage of heat treatment, together with the activation energy of inactivation, its standard deviation, and the variation in the rate constant. The thermal stability of immobilized TPI is influenced by the pH, the ion concentration, and substances bindig specifically to the enzyme molecule. The presence of substrate does not alter the thermal stability appreciably, which indicates that protection of the active centre itself does not increase the stability. In contrast, the presence of phosphate ions leads to stabilization not only in the active centre, but also through coupling to the surface of the TPI molecule.

7. The conditions of enzyme immobilization influence the orientation of the enzyme molecule on covalent binding. The conformation and hence the thermal

stability of the enzymes immobilized on the Akrilex C—100 support are changed in different ways by the chemical and steric structures of the water-soluble disubstituted carbodiimides present as activators during immobilization. For carbodiimide derivatives with low bulk the amino groups of the enzyme become more accessible sterically on immobilization. Consequently, the immobilized enzymes have high thermal stability, and the standard deviation of the activation energy indicates a high degree of heterogeneity.

8. The immobilized enzymes were used for continuous analytical measurements in enzyme reactors, and in preparative operations. The concentrations of FDP and of glucose were determined in a flow injection system with an immobilized aldolase-TPI-GDH enzyme reactor and in a GOD-POD reactor, respectively. The latter system is suitable for the analysis of blood samples containing low glucose concentrations, such as the blood serum of fish in the resting state.

9. Dihydroxyacetone phosphate was prepared from FDP in a laboratory-scale, immobilized aldolase-TPI enzyme reactor. The reactor functioned with a maximum conversion of 70%, for 1 month, within any appreciable activity decrease.

10. Gluconic acid was prepared under regulated experimental conditions in an experimental GOD-catalase batch reactor. The productivity was 100—110 mmol/g enzyme/hour.

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